SIXTH EDITION

Principles of Instrumental Analysis

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1D CALIBRATION OF INSTRUMENTAL METHODS

A very important part of all analytical procedures is the calibration and standardization process. *Calibration* determines the relationship between the analytical response and the analyte concentration. Usually this is determined by the use of *chemical standards*.

Almost all analytical methods require some type of calibration with chemical standards. Gravimetric methods and some coulometric methods (Chapter 24) are among the few *absolute* methods that do not rely on calibration with chemical standards. Several types of calibration procedures are described in this section.

1D-1 Comparison with Standards

Two types of comparison methods are described here, the direct comparison technique and the titration procedure.

Direct Comparison

Some analytical procedures involve comparing a property of the analyte (or the product of a reaction with the analyte) with standards such that the property being tested matches or nearly matches that of the standard. For example, in early colorimeters, the color produced as the result of a chemical reaction of the analyte was compared with the color produced by reaction of standards. If the concentration of the standard was varied by dilution, for example, it was possible to obtain a fairly exact color match. The concentration of the analyte was then equal to the concentration of the standard after dilution. Such a procedure is called a *null comparison* or *isomation method*.[2]

Titrations

Titrations are among the most accurate of all analytical procedures. In a titration, the analyte reacts with a standardized reagent (the titrant) in a reaction of known stoichiometry. Usually the amount of titrant is varied until chemical equivalence is reached, as indicated by the color change of a chemical indicator or by the change in an instrument response. The amount of the standardized reagent needed to achieve chemical equivalence can then be related to the amount of analyte present. The titration is thus a type of chemical comparison.[3]

1D-2 External-Standard Calibration

An *external standard* is prepared separately from the sample. By contrast, an *internal standard* is added to the sample itself. External standards are used to calibrate instruments and procedures when there are no interference effects from matrix components in the analyte solution. A series of such external standards containing the analyte in known concentrations is prepared. Ideally, three or more such solutions are used in the calibration process. However, in some routine analyses, two-point calibrations can be reliable.

Calibration is accomplished by obtaining the response signal (absorbance, peak height, peak area) as a function of the known analyte concentration. A calibration curve is prepared by plotting the data or by fitting them to a suitable mathematical equation, such as the slope-intercept form used in the method of linear least squares. The next step is the prediction step, where the response signal is obtained for the sample and used to *predict* the unknown analyte concentration, c_x, from the calibration curve or best-fit equation. The concentration of the analyte in the original bulk sample is then calculated from c_x by applying the appropriate dilution factors from the sample preparation steps.

The Least-Squares Method

A typical calibration curve is shown in Figure 1-8 for the determination of isooctane in a hydrocarbon sample. Here, a series of isooctane standards was injected into a gas chromatograph, and the area of the isooctane peak was obtained as a function of concentration. The ordinate is the dependent variable, peak area, and the abscissa is the independent variable, mole percent (mol %) of isooctane. As is typical and usually desirable, the plot approximates a straight line. Note, however, that because of the indeterminate errors in the measurement process, not all the data fall exactly on the line. Thus, the investigator must try to draw the "best" straight line among the data points. *Regression analysis* provides the means for objectively obtaining such a line

Tutorial: Learn more about **calibration**.

[2] See, for example, H. V. Malmstadt and J. D. Winefordner, *Anal. Chim. Acta*, **1960**, *20*, 283; L. Ramaley and C. G. Enke, *Anal. Chem.*, **1965**, *37*, 1073.

[3] See D. A. Skoog, D. M. West, F. J. Holler, and S. R. Crouch, *Fundamentals of Analytical Chemistry*, 8th ed., Belmont, CA: Brooks/Cole, 2004, Chaps. 13–17.

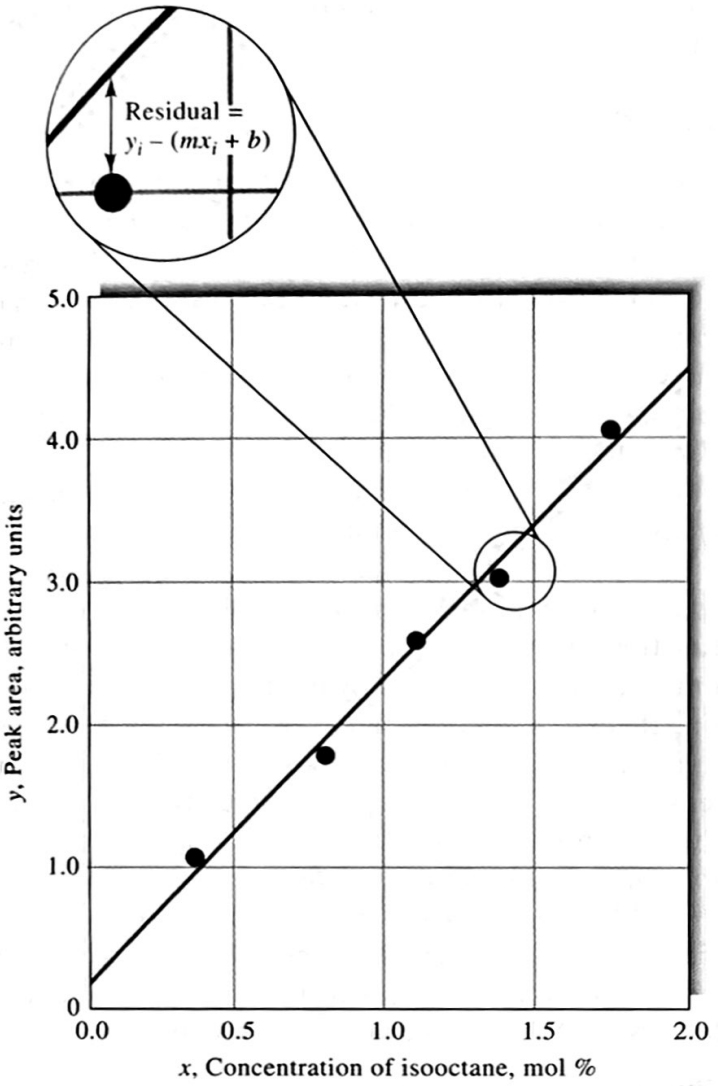

FIGURE 1-8 Calibration curve for the determination of isooctane in a hydrocarbon mixture. The residual is the difference between an experimental data point y_i and that calculated from the regression model, $mx_i + b$, as shown in the insert.

and also for specifying the uncertainties associated with its subsequent use. The uncertainties are related to the *residuals* shown in Figure 1-8, which are a measure of how far away from the best straight line the data points lie. The *method of least squares* (see Appendix 1, Section a1D) is often applied to obtain the equation for the line.[4]

The method of least squares is based on two assumptions. The first is that there is actually a linear relationship between the measured response y and the standard analyte concentration x. The mathematical relationship that describes this assumption is called the *regression model*, which may be represented as

$$y = mx + b$$

where b is the y intercept (the value of y when x is zero) and m is the slope of the line (see Figure 1-8). We also assume that any deviation of the individual points from the straight line arises from error in the *measurement*. That is, we assume there is no error in the x values of the points (concentrations). Both of these assumptions are appropriate for many analytical methods, but bear in mind that whenever there is significant uncertainty in the x data, basic linear least-squares analysis may not give the best straight line. In such a case, a more complex *correlation analysis* may be necessary. In addition, basic least-squares analysis may not be appropriate when the uncertainties in the y values vary significantly with x. In this case, it may be necessary to apply different weighting factors to the points and perform a *weighted least-squares analysis*.[5]

In cases where the data do not fit a linear model, nonlinear regression methods are available.[6] Some of these use polynomial models or multiple regression procedures. There are even computer programs that will find a model that describes a set of experimental data from an internal or user-defined set of equations.[7]

The slope m and intercept b of the linear least-squares line are determined as in Equations a1-34 and a1-35 of Appendix 1. For determining an unknown concentration c_x from the least-squares line, the value of the instrument response y_c is obtained for the unknown, and the slope and intercept are used to calculate the unknown concentration c_x as shown in Equation 1-1.

$$c_x = \frac{y_c - b}{m} \tag{1-1}$$

The standard deviation in concentration s_c can be found from the *standard error of the estimate* s_y, also called the *standard deviation about regression*, as given in Equation 1-2:

$$s_c = \frac{s_y}{m}\sqrt{\frac{1}{M} + \frac{1}{N} + \frac{(\bar{y}_c - \bar{y})^2}{m^2 S_{xx}}} \tag{1-2}$$

[4]For a discussion of using spreadsheets in linear regression analysis, see S. R. Crouch and F. J. Holler, *Applications of Microsoft® Excel in Analytical Chemistry*, Belmont, CA: Brooks/Cole, 2004, Chap. 4.

[5]See P. R. Bevington and D. K. Robinson, *Data Reduction and Error Analysis for the Physical Sciences*, 3rd ed., New York: McGraw-Hill, 2002.

[6]J. L. Devore, *Probability and Statistics for Engineering and the Sciences*, 6th ed., Pacific Grove, CA: Duxbury Press at Brooks/Cole, 2004.

[7]See, for example, *TableCurve*, Systat Software, Point Richmond, CA.

where M is the number of replicate results, N is the number of points in the calibration curve (number of standards), $\bar{y}_c$ is the mean response for the unknown, and $\bar{y}$ is the mean value of y for the calibration results. The quantity S_{xx} is the sum of the squares of the deviations of x values from the mean as given in Equation a1-31 of Appendix 1.

Errors in External-Standard Calibration

When external standards are used, it is assumed that the same responses will be obtained when the same analyte concentration is present in the sample and in the standard. Thus, the calibration functional relationship between the response and the analyte concentration must apply to the sample as well. Usually, in a determination, the raw response from the instrument is not used. Instead, the raw analytical response is corrected by measuring a *blank*. An *ideal blank* is identical to the sample but without the analyte. In practice, with complex samples, it is too time-consuming or impossible to prepare an ideal blank and a compromise must be made. Most often a real blank is either a *solvent blank*, containing the same solvent in which the sample is dissolved, or a *reagent blank*, containing the solvent plus all the reagents used in sample preparation.

Even with blank corrections, several factors can cause the basic assumption of the external-standard method to break down. Matrix effects, due to extraneous species in the sample that are not present in the standards or blank, can cause the same analyte concentrations in the sample and standards to give different responses.[8] Differences in experimental variables at the times at which blank, sample, and standard are measured can also invalidate the established calibration function. Even when the basic assumption is valid, errors can still occur because of contamination during the sampling or sample preparation steps.

Also, systematic errors can occur during the calibration process. For example, if the standards are prepared incorrectly, an error will occur. The accuracy with which the standards are prepared depends on the accuracy of the gravimetric and volumetric techniques and equipment used. The chemical form of the standards must be identical to that of the analyte in the sample; the state of oxidation, isomerization, or complexation of the analyte can alter the response. Once prepared, the concentration of the standards can change because of decomposition, volatilization, or adsorption onto container walls. Contamination of the standards can also result in higher analyte concentrations than expected. A systematic error can occur if there is some bias in the calibration model. For example, errors can occur if the calibration function is obtained without using enough standards to obtain good statistical estimates of the parameters.

Random errors can also influence the accuracy of results obtained from calibration curves, as illustrated in Figure 1-9. The uncertainty in the concentration of analyte s_c' obtained from a calibration curve is lowest when the response is close to the mean value $\bar{y}$. The point $\bar{x}, \bar{y}$ represents the centroid of the regression line. Note that measurements made near the center of the curve will give less uncertainty in analyte concentration than those made at the extremes.

Multivariate Calibration

The least-squares procedure just described is an example of a univariate calibration procedure because only one response is used per sample. The process of relating multiple instrument responses to an analyte or a mixture of analytes is known as *multivariate calibration*. Multivariate calibration methods[9] have become quite popular in recent years as new instruments become available that produce multidimensional responses (absorbance of several samples at multiple wavelengths, mass spectrum of chromatographically separated components, etc.). Multivariate calibration methods are very powerful. They can be used to simultaneously determine multiple components in mixtures and can provide redundancy in measurements to improve precision because repeating a measurement N times provides a $\sqrt{N}$ improvement in the precision of the mean value (see Appendix 1, Section a1B-1). They can also be used to detect the presence of interferences that would not be identified in a univariate calibration.

1D-3 Standard-Addition Methods

Standard-addition methods are particularly useful for analyzing complex samples in which the likelihood of matrix effects is substantial. A standard-addition

[8] The *matrix* includes the analyte and other constituents, which are termed *concomitants*.

[9] For a more extensive discussion, see K. R. Beebe, R. J. Pell, and M. B. Seasholtz, *Chemometrics: A Practical Guide*, New York: Wiley, 1998, Chap. 5; H. Martens and T. Naes, *Multivariate Calibration*, New York: Wiley, 1989.

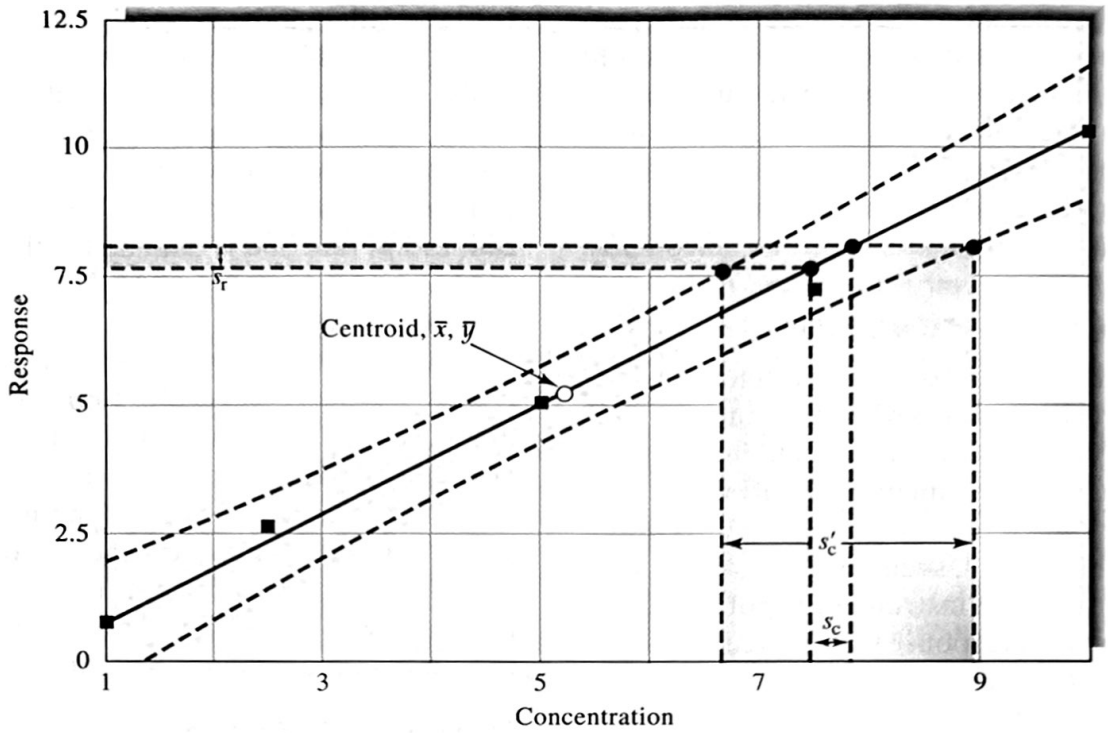

FIGURE 1-9 Effect of calibration curve uncertainty. The dashed lines show confidence limits for concentrations determined from the regression line. Note that uncertainties increase at the extremities of the plot. Usually, we estimate the uncertainty in analyte concentration only from the standard deviation of the response. Calibration curve uncertainty can significantly increase the uncertainty in analyte concentration from s_c to s_c'.

method can take several forms.[10] One of the most common forms involves adding one or more increments of a standard solution to sample aliquots containing identical volumes. This process is often called *spiking* the sample. Each solution is then diluted to a fixed volume before measurement. Note that when the amount of sample is limited, standard additions can be carried out by successive introductions of increments of the standard to a single measured volume of the unknown. Measurements are made on the original sample and on the sample plus the standard after each addition. In most versions of the standard-addition method, the sample matrix is nearly identical after each addition, the only difference being the concentration of the analyte or, in cases involving the addition of an excess of an analytical reagent, the concentration of the reagent. All other constituents of the reaction mixture should be identical because the standards are prepared in aliquots of the sample.

Assume that several aliquots V_x of the unknown solution with a concentration c_x are transferred to volumetric flasks having a volume V_t. To each of these flasks is added a variable volume V_s of a standard solution of the analyte having a known concentration c_s. Suitable reagents are then added, and each solution is diluted to volume. Instrumental measurements are then made on each of these solutions and corrected for any blank response to yield a net instrument response S. If the blank-corrected instrument response is proportional to concentration, as is assumed in the standard-addition method, we may write

$$S = \frac{kV_s c_s}{V_t} + \frac{kV_x c_x}{V_t} \tag{1-3}$$

where k is a proportionality constant. A plot of S as a function of V_s is a straight line of the form

$$S = mV_s + b$$

where the slope m and the intercept b are given by

$$m = \frac{kc_s}{V_t}$$

[10] See M. Bader, *J. Chem. Educ.*, **1980**, *57*, 703.